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CEACAM6 promotes tumor angiogenesis and vasculogenic mimicry in gastric cancer *via* FAK signaling

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ABSTRACT

CEACAM6 is a member of glycosylphosphatidylinositol-linked immunoglobulin superfamily that is implicated in a variety of human cancers. In our previous study, we reported that CEACAM6 was overexpressed in gastric cancer tissues and promoted cancer metastasis. The purpose of this study is to determine the role of CEACAM6 in tumor angiogenesis and mimicry formation. We found that overexpressed CEACAM6 promoted tubule formation dependent on HUVEC cells and vasculogenic mimicry formation of gastric cancer cells; opposing results were achieved in CEACAM6-silenced groups. Moreover, we found that mosaic vessels formed by HUVEC cells and gastric cancer cells were observed *in vitro* by 3D-culture assay. Overexpressed CEACAM6 in gastric cancer cells promoted tumor growth, VEGF expression and vasculogenic mimicry structures formation *in vivo*. In accordance with these observations, we found that phosphorylation of FAK and phosphorylation of paxillin were up-regulated in CEACAM6-overexpressing gastric cancer cells, and FAK inhibitor Y15 could reduce tubule and vasculogenic mimicry formation. These findings suggest that CEACAM6 promotes tumor angiogenesis and vasculogenic mimicry formation *via* FAK signaling in gastric cancer and CEACAM6 may be a new target for cancer anti-vascular treatment.

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1. Introduction

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), lacking transmembrane and intracellular domains, is a glycosylphosphatidylinositol (GPI)-linked immunoglobulin superfamily member that is overexpressed in a variety of human tumors [1–4]. CEACAM6 is co-localized to microdomains in the plasma membrane with itself and other CEACAMs [5,6], they can form co-clusters activating integrin signaling and its downstream signaling cascades, such as FAK, PI3K/AKT, and MAPK [7–9]. GC is one of the most common malignant tumors and a major health burden worldwide [10,11]. New treatments to this malignancy are urgently needed.

Tumor metastasis is the leading cause of cancer-related death but its mechanisms remain unknown. Tumor angiogenesis is a crucial aspect in the development of primary tumors or secondary tumors, particularly when tumors diameter approximates 2 mm [12]. While new blood vessels include not only angiogenesis formed by endothelial cells, but also VM tubules formed by malignant cancer cells. VM tubule is an endothelium-independent pattern and first reported in melanoma study [13]. VM is strongly involved in a variety of malignant human tumors including gastric cancer [14,15]. Red blood cells and nutrients, contributing to cancer growth and metastasis, can be transferred from endothelial vessels to VM vessels. VM contributes to tumor metastasis, poor prognosis, a poor 5-year overall survival and increases patient mortality [16,17]. However, traditional anti-vascular treatment may contribute to the progression of cancer by inducing hypoxia which stimulates VM formation [18]. Novel anti-vascular therapeutic approaches to cancer should be designed. We recently reported that CEACAM6 is up-regulated in GC tissues and promotes GC cell metastasis *via* c-SRC signaling [19]. FAK signaling is associated with tumor angiogenesis as well as cancer metastasis. The aim of this study is to determine the role of CEACAM6 in angiogenesis and VM formation in GC, and to explore its potential downstream signaling targets, such as FAK signaling.

2. Materials and methods

2.1. Cell lines

The human GC cell lines SGC-7901, MKN-45, MKN-28 and human umbilical vein endothelial (HUVEC) cells were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of

Abbreviations: CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; GC, gastric cancer; VM, vasculogenic mimicry; HUVEC, human umbilical vein endothelial; FAK, focal adhesion kinase

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Sciences. Cells were cultured at 37 °C in 5% CO_2 and saturation humidity in RPMI-1640 medium containing 10% fetal bovine serum.

2.2. Vector construction and transfection

The CEACAM6 primer sequences were 5′-CCGGAATTCCCATGGGAC CCCCCTCAGCCC-3′ (forward) and 5′-TCCCCCGGGGCTATATCAGAGCCA CCCTGG-3′ (reverse). Full-length CEACAM6 cDNA was obtained by RT-PCR from total RNA extracted from GC samples. Then we assembled a pIRES2-eGFP-CEACAM6 construct by inserting CEACAM6 cDNA into pIRES2-eGFP vector. Moreover pIRES2-eGFP-CEACAM6 or pIRES2-eGFP vector was transfected into SGC-7901 and MKN-45 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) in accordance with the manufacturer's protocol. Stable clones were selected by continuous treatment with G418 (1.2 mg/ml; Gibco, New York, USA).

2.3. Lentiviral vector construction

Based on human CEACAM6 gene data, Shanghai Novobio Scientific Co., Ltd designed and synthesized a pair of oligonucleotide sequences and negative control sequences. shRNA sequences of CEACAM6 or negative control were as follows: 5′-CACCGCCGGACAGTTCCATGTATA CGAATATACATGGAACTGTCCGG-3′ (forward), and 5′-AAAACCGGACAG TTCCATGTATATTCGTATACATGGAACTGTCCGGC-3′ (reverse); Negative control, 5′-CACCGCTACACAAATCAGCGATTTCGAAAAATCGCTGATTTG TGTAG-3′ (forward), and 5′-AAAACTACACAAATCAGCGATTTTTCGAAA TCGCTGATTTGTGTAGC-3′ (reverse). CEACAM6 shRNA was subcloned into pL/shRNA/F lentiviral vector to obtain a pL/shRNA/shR-CEACAM6 construct. Then pL/shRNA/shR-CEACAM6 or control lentiviral vector was transfected into MKN-28, SGC-7901-CEACAM6 and MKN-45-CEACAM6 GC cells. Stably transfected cells were selected by treatment with 5 μg/ml blasticidin and were used for identification and further research.

2.4. Western blotting

Cells and tumor samples were harvested and lysed using RIPA buffer (Solarbio, Beijing, China) containing 1% PMSF protease inhibitors. The total protein concentration was measured by a BCA assay kit (Pierce, Rockford, USA). Equivalent amounts of protein were separated by 10% SDS-PAGE, and the resolved proteins transferred to PVDF membranes. The membranes were blocked with 5% skim milk for 2 h and then incubated with primary antibodies overnight at 4 °C. Primary antibodies were as follows: CEACAM6 (9A6;1:500; Abcam, USA), paxillin (Cell Signaling Technology (CST), USA), p-paxillin (Tyr118) (CST, USA), FAK (CST, USA), p-FAK (Tyr925) (CST, USA), p-FAK (Tyr576/577) (CST, USA), p-FAK (Tyr397) (CST, USA) and GAPDH (Abcam, USA). Membranes were then incubated with secondary antibody for 2 h at room temperature and were visualized using an enhanced chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ, USA) according to the manufacturer's protocol.

2.5. Semi-quantitative RT-PCR assay

RNA was extracted from gastric cancer cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using the reverse transcription kit (Promega, Madision, WI, USA). Primers of CEACAM6 were 5′-TCAATGGGACGTTC CAGCAAT-3′ (forward) and 5′-CACTCCAATCGTGATGCCGA-3′ (reverse). Primers of GAPDH were 5′-GGACCTGACCTGCCGTCTAG-3′ (forward) and 5′-GTAGCCCAGGATGCCCTTGA-3′ (reverse). Then PCR reactions were carried out and the products of PCR were separated by 2% agarose gel (Sigma, St Louis, MO, USA). The GAPDH was used as an internal control to verify the absence of significant variation in the cDNA levels in the samples.

2.6. Endothelial tube formation assay

HUVEC cells were cultured in tumor supernatant with or without FAK inhibitor 14 (1,2,4,5-benzenetetramine tetrahydrochloride (Y15); Cayman, Michigan, USA) and plated in 96-well plate coated with 50 μl matrigel (BD Bioscience, CA, USA) at the concentration 3×10^4 cells/well. Tumor supernatant was collected from CEACAM6-overexpressing groups and CEACAM6-silenced groups after cultured 24 h in RPMI-1640. After 12 h incubation at 37 °C with 5% CO_2, tubules were photographed by microscopy and evaluated by Image Pro Plus software.

2.7. Cell migration assay

For cell migration assay, a total number of 1×10^5 HUVEC cells were added in serum-free RPMI-1640 with or without tumor supernatant and plated in transwell chambers (8 μm for 24-well plate; Corning Costar, NY, USA) according to the manufacturer's protocols. After 12 h incubation, HUVEC cells were fixed by 10% formalin and stained by 0.5% crystal violet. Finally, HUVEC cells in the lower chamber were photographed and counted by inverted microscopy.

2.8. Cell proliferation assay

Cell proliferation was monitored by Cell Counting Kit-8 (CCK-8). HUVEC cells were cultured in tumor supernatant and plated in 96-well plate at the concentration 2000 cells/well. Cell proliferation was measured every 24 h for 5 days after adding CCK-8 2 h at the absorbance 450 nm using a Epoch Microplate Spectrophotometer (Bio Tek).

2.9. Three-dimensional (3D) culture and mosaic vessel assays

For 3D-culture and mosaic vessel assays, matrigel was thawed overnight at 4 °C. Then 100 μl matrigel was used to coat the wells of a 24-well plate and was allowed to polymerize for 2 h at 37 °C. MKN-45-CEACAM6, which carried with eGFP label, and HUVEC cells without eGFP label were mixed according to 2:8 and 5:5. Tumor cells or mixed cells (1×10^5 cells) were cultured in complete growth medium with or without FAK Inhibitor Y15 and were plated into 24-well at the concentration 1×10^5 cells/well. Tubules were photographed by an AMG fluorescence microscope after 24 h incubation at 37 °C with 5% CO_2 and were evaluated by Image Pro Plus software.

2.10. VM assay

Serum-free RPMI-1640 medium and matrigel were mixed according to 2:1. The mixture was then seeded onto 16 mm glass cover slides in 6-well plate and was allowed to polymerize for 2 h at 37 °C. Then 1×10^5 GC cells were seeded onto each plate and cultured for 3 days at 37 °C with 5% CO_2. Moreover, GCs were fixed by 4% paraformaldehyde and stained by PAS stain according to the manufacturer's protocols. After counterstained with hematoxylin, slides were dehydrated and covered.

2.11. Immunohistochemical and histochemical double-staining methods

Sections of 4 μm thick were cut from paraffin-embedded nude mice tumor and human cancer tissue blocks and then deparaffinized and rehydrated. Immunohistochemical staining of sections was performed according to the DAKO protocol, using primary antibody mouse anti-CEACAM6 (1:100; Abcam), primary antibody rabbit anti-VEGF (1:100; Abcam) and mouse anti-CD34 (1:100; Santa Cruz Biotechnology) at 37 °C for 2 h. After stained by anti-CD34 and visualized by diaminobenzidine, sections were incubated with PAS (Leagene, Beijing, China) according to the protocol. CD34 positive vessels indicated blood vessels in tissues, and CD34 negative, PAS positive vessels were defined as VM.

2.12. Nude mice tumorigenesis

SGC-7901-CEACAM6 and SGC-7901-NC (1×10^6 cells) were subcutaneously injected into 4-week-old male BALB/c nude mice (Institute of Zoology, China Academy of Sciences). Tumor nodules were measured every 7 days, and were calculated using the formula: tumor volume = (Width^2 × Length) / 2. Mice were killed 5 weeks after injection. Tumors were weighed and fixed for immunohistochemistry staining.

2.13. Statistical analysis

Data are shown as mean ± SD. Differences between experimental groups were assessed by the Student's t test or one-way ANOVA. A two-tailed value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS 19.0 software (SPSS Inc).

3. Results

3.1. CEACAM6 promotes tubular formation of HUVEC cells

CEACAM6 overexpression and knockdown in GC cells were confirmed by western blotting and RT-PCR (Fig. 1A; Fig. S1.A). Then tumor supernatant was collected from SGC-7901-CEACAM6, SGC-7901-CEACAM6/sh, MKN-45-CEACAM6, MKN-45-CEACAM6/sh, MKN-28-CEACAM6/sh and their control groups to culture HUVEC cells. Thirty thousand HUVEC cells were plated into 96-well plate coated with

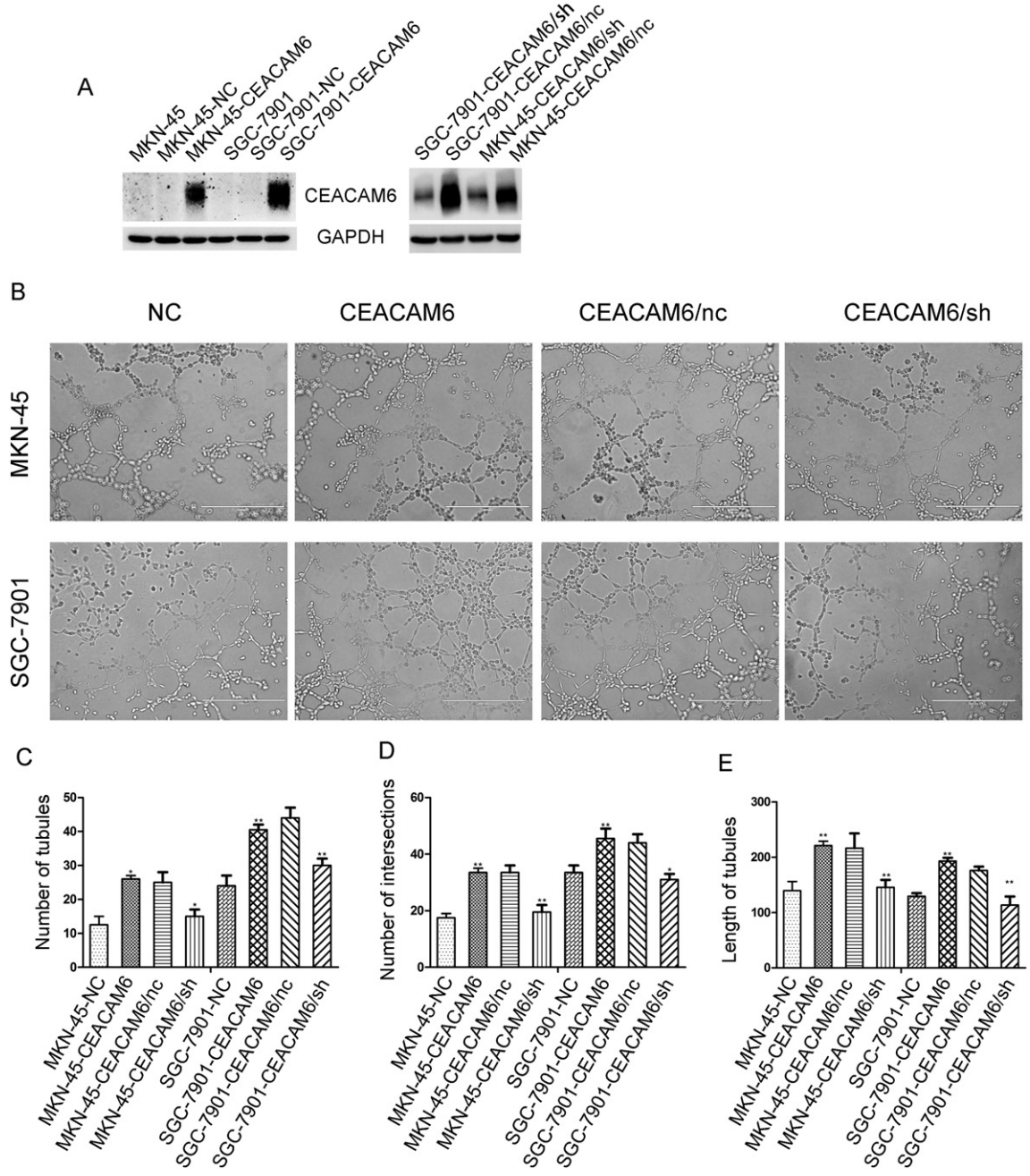

Fig. 1. Effect of CEACAM6 on tubular formation *in vitro*. (A) Overexpression and suppression of CEACAM6 in GC cells were confirmed at the protein level by western blotting. (B) Rich tubules were observed in the SCG-7901-CEACAM6 and MKN-45-CEACAM6 groups compared with the control groups; whereas the SCG-7901-CEACAM6/sh and MKN-45-CEACAM6/sh groups formed less tubules than the SCG-7901-CEACAM6/nc and MKN-45-CEACAM6/nc groups (200×). (C–E) Bar charts show numbers of tubules (C), numbers of intersections (D) and mean tubular lengths (E) between different groups. $^*P < 0.05$; $^{**}P < 0.01$. Data are represented as mean ± SD of three independent experiments.

matrigel and incubated for 12 h at 37 °C with 5% CO_2. Then we observed increased tubules forming ability in CEACAM6-overexpressing groups compared with the control groups (Fig. 1B), such as the number of tubules (Fig. 1C), number of intersections (Fig. 1D), and mean length of tubules (Fig. 1E). Opposing results were achieved in CEACAM6-silenced groups compared with the control groups (Fig. 1, Fig. S1.C).

3.2. CEACAM6 promotes HUVEC cell proliferation and migration

Tubule formation of HUVEC cells was correlated with HUVEC cell proliferation and migration. After HUVEC cells incubating in tumor supernatant for 24 h, HUVEC cell proliferation was increased in CEACAM6-overexpressing groups and decreased in CEACAM6-silenced groups compared with control groups (Fig. 2A, B). A similar tendency with HUVEC cell proliferation was observed in HUVEC cell migration. The migration ability of HUVEC cells was enhanced in CEACAM6-overexpressing groups and inhibited in CEACAM6-silenced groups compared with control groups (Fig. 2C, D; Fig. S1.D). Thus, these results suggested CEACAM6 promoted tumor angiogenesis in GC by stimulating HUVEC cell proliferation and migration.

3.3. CEACAM6 promotes VM formation in GC cells

VM tubules were defined as vessels formed by malignant tumor cells, which was different from blood vessels formed by HUVEC cells. More VM structures were observed in CEACAM6-overexpressing groups than the control groups (Fig. 3A). The opposing result was obtained in CEACAM6-silenced groups compared with the control groups (Fig. 3A; Fig. S1.B). And the VM forming ability was evaluated by the number of tubules (Fig. 3B), number of intersections (Fig. 3C), and mean length of tubules (Fig. 3D).

Furthermore, to illustrate the effect of CEACAM6 on VM in GC cells, another assay, periodic acid-Schiff (PAS) staining, was performed. As well as 3D-culture assay, PAS staining showed overexpressed CEACAM6 promoted VM formation in GC cells (Fig. 4). In conclusion, CEACAM6 could enhance the VM forming ability of GC cells.

3.4. GC cells and HUVEC cells form mosaic vessels in vitro

Nutrition for tumor growth is supplied by HUVEC and VM vessels. Mosaic vessels serve as a bridge to transfer nutrition for HUVEC and VM vessels. To verify that mosaic vessels exist *in vitro*, GC cells were mixed with HUVEC cells according to the proportion 2:8 and 5:5 at the total number 1×10^5, then the mixture cells were plated into 24-well plate coated with matrigel. GC cells labeled with eGFP showed green image under the fluorescence microscope (Fig. 5A). However, HUVEC cells without eGFP showed gray image under the fluorescence microscope (Fig. 5B). Mosaic vessels were observed in the mixture cells composed of GC cells and HUVEC cells according to the proportion 2:8 (Fig. 5C) and 5:5 (Fig. 5D). These findings confirmed that GC cells and HUVEC cells were able to form mosaic vessels to supply nutrition for tumor growth.

3.5. CEACAM6 upregulates phosphorylated FAK and paxillin in GC cells

FAK signaling is associated with tumor angiogenesis as well as cancer metastasis. Our previous study showed that CEACAM6 promoted GC metastasis *in vivo* and *in vitro* [19]. In this study, overexpressed

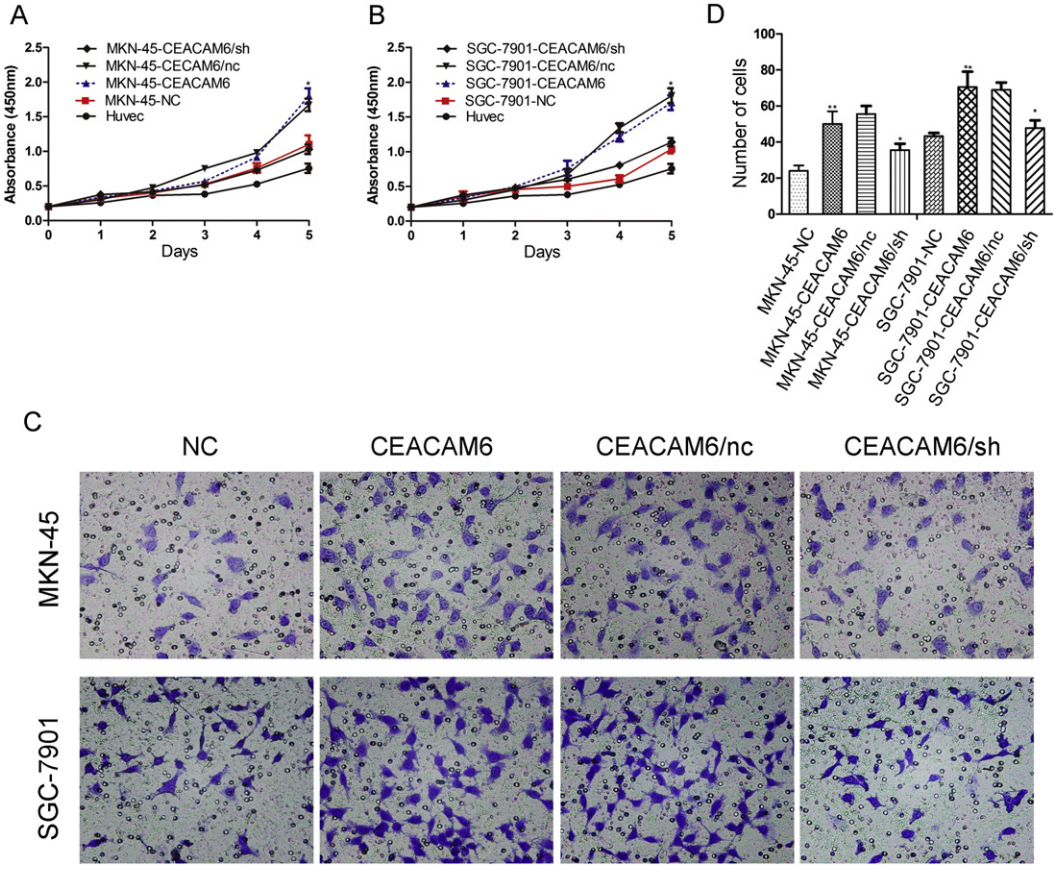

Fig. 2. Effects of CEACAM6 on migration and proliferation of HUVECs. The tumor supernatant from SCG-7901-CEACAM6, SCG-7901-CEACAM6/sh, MKN-45-CEACAM6, MKN-45-CEACAM6/sh and their control groups was collected to treat HUVEC cells, and we next examined the migration and proliferation of HUVECs. (A, B) The proliferation of HUVECs was increased in CEACAM6-overexpressing groups compared with the control groups, and opposing result was observed in CEACAM6-silenced groups. (C) Overexpressed CEACAM6 promoted the migration of HUVECs, whereas silenced CEACAM6 reduced the migration of HUVECs (200×). (D) Histograms showed the numbers of migration cells. $^*P < 0.05$; $^{**}P < 0.01$. Data are represented as mean ± SD of three independent experiments.

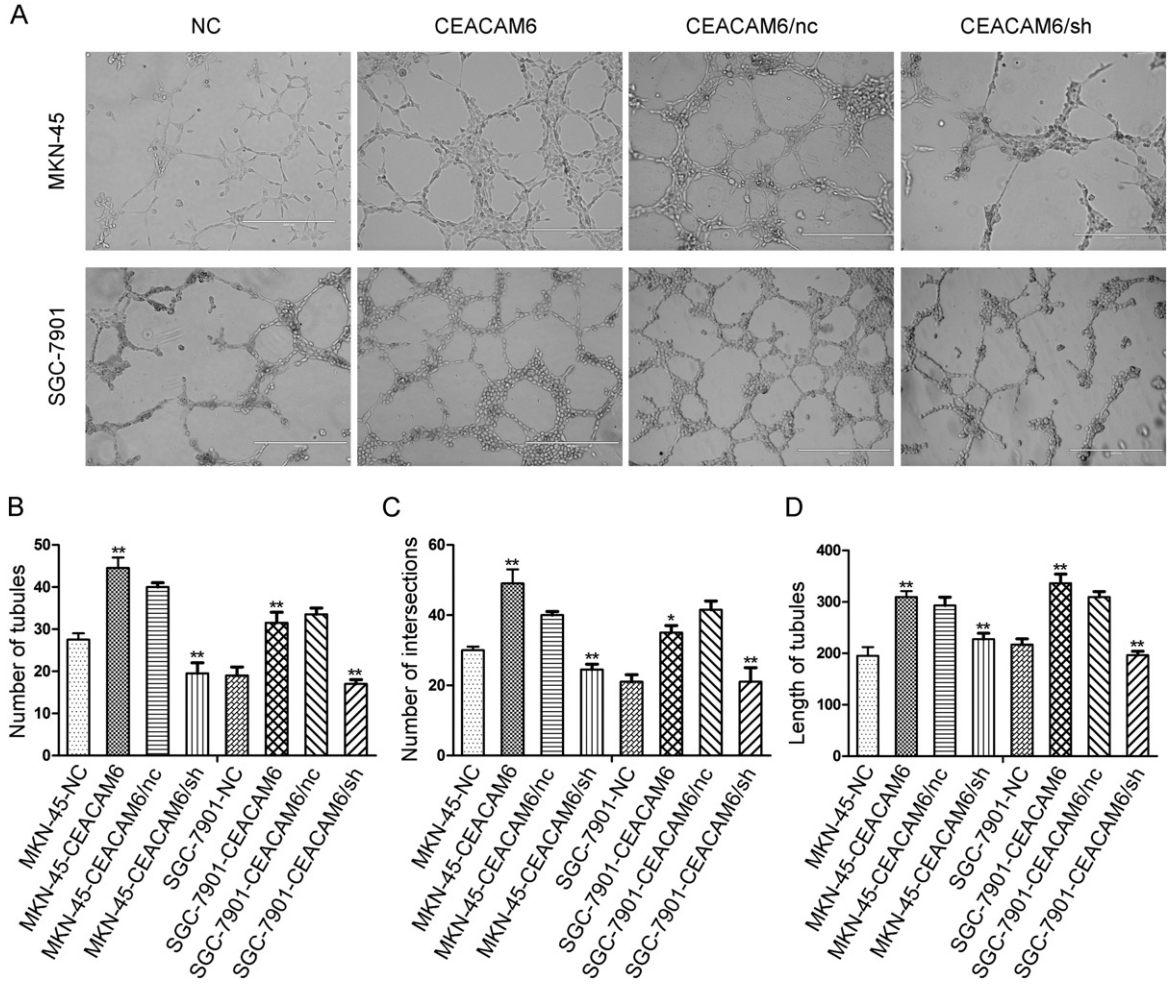

Fig. 3. Effect of CEACAM6 on VM in GC cells on matrigel. (A) More vessel-like structures defined as VM were formed in CEACAM6-overexpressing groups, on the contrary the converse results were observed in CEACAM6-silenced groups compared with the control groups, respectively (200×). The numbers of tubules (B), numbers of intersections (C) and mean tubular lengths (D) between different groups. $^{*}P < 0.05$; $^{**}P < 0.01$. Data are represented as mean ± SD of three independent experiments.

CEACAM6 in MKN-45 and SGC-7901 (Fig. 6A) increased Tyr925, Tyr576/577, and Tyr397 phosphorylation of FAK and phosphorylation of paxillin (Tyr118) (Fig. 6B, C). Opposing results were observed in CEACAM6-silenced groups compared with control groups (Fig. 6A, B, C). Meanwhile, we found that p-FAK was increased in human GC tissues which overexpressed CEACAM6 (Fig. S2.C). Interestingly, tubule and VM formation was decreased after treated with FAK inhibitor Y15 in SGC-7901-CEACAM6 GC cells. Collectively, these findings suggested that CEACAM6 promoted tumor angiogenesis and VM formation *via* FAK signaling.

3.6. CEACAM6 promotes tumorigenesis and VM in vivo

The effect of CEACAM6 on tumorigenesis was examined by subcutaneously injecting SGC-7901-NC and SGC-7901-CEACAM6 GC cells into nude mice. We observed that overexpressed CEACAM6 promoted tumor growth compared with the control groups (Fig. 7A). The average tumor volume was much larger in CEACAM6-overexpressing groups than that in control groups (Fig. 7B). Moreover, the weight of tumors was increased in SGC-7901-CEACAM6 groups compared with the control groups (1.55 ± 0.18 g vs 1.05 ± 0.22 g, $P < 0.05$; Fig. 7C). Enough nutrition and red blood cells from increasing vessels may be the interpretation about the above findings.

Furthermore, tumor sections from nude mice mold were stained by VEGF and CD34/PAS double staining. We observed that VEGF expression was increased in SGC-7901-CEACAM6 groups compared with SGC-7901-NC groups (Fig. 7D). And more VM structures with or without red blood cells were observed in SGC-7901-CEACAM6 groups compared with SGC-7901-NC groups (Fig. 7E, F). The IHC showed that more CD34 positive expression was obtained in GC tissues than in non-tumor tissues (Fig. S2.B). This tendency was congruity with CEACAM6 expression in GC samples (Fig. S2.A). Meanwhile, the level of p-FAK (397) extracted from tumor of nude mice was increased in SCG-7901-CEACAM6 groups compared with the control groups. These findings *in vivo* were consistent with the results *in vitro*. These results suggested that CEACAM6 promoted gastric tumor growth *via* stimulating VM formation *in vivo*.

4. Discussion

We recently reported that CEACAM6 is overexpressed in GC tissues and promotes GC tumor invasion and migration [19]. It is important to note that although CEACAM6 lacks transmembrane and intracellular domains, it does regulate a variety of signaling pathways promoting cancer metastasis, anoikis resistance, chemoresistance, and inhibiting differentiation [3,19–22]. Cancer metastasis is correlated with tumor angiogenesis. Based on these observations, the purpose of this study is to determine the role of CEACAM6 in mediating tumor angiogenesis and VM formation in GC.

Interestingly, we observed that CEACAM6 promoted tubule formation dependent on HUVEC cells in this study. As an attempt to understand the events which promoted tubule formation *via* CEACAM6,

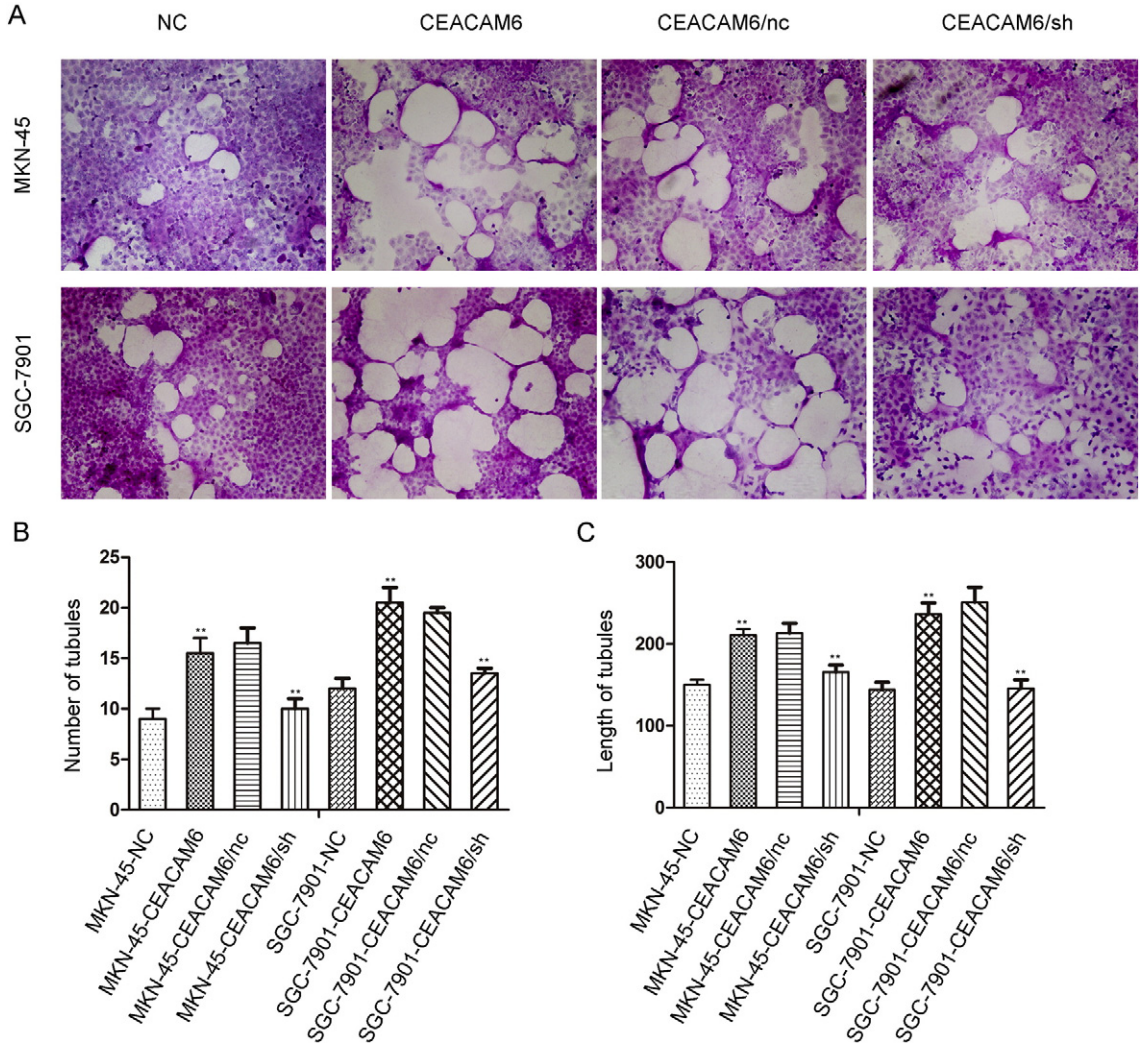

Fig. 4. Effect of CEACAM6 on VM in GC cells by PAS staining. (A) PAS staining was performed to examine VM structures in GC cells. Consistent with the GC cells tubule formation on matrigel, more PAS positive VM structures were detected in CEACAM6-overexpressing groups compared with the control groups. However, the VM structures of CEACAM6-silenced groups were less than the control groups (200×). Histograms showed the numbers of tubules (B) and mean tubular lengths (C) between different groups. $^{*}P < 0.05$; $^{**}P < 0.01$. Data are represented as mean ± SD of three independent experiments.

HUVEC cell proliferation and migration assays were performed after HUVEC cells incubating in tumor supernatant. We found that CEACAM6 was able to promote HUVEC cell proliferation and migration. The increasing proliferation and migration of HUVEC cells contributed to tubule formation in GC. HUVEC cells do not express CEACAMs. How can CEACAM6 promote tubule HUVEC cell proliferation and migration? Thus, there must be some factors, secreted by GC cells, in tumor supernatant to stimulate proliferation and migration of HUVEC cells rather than CEACAM6 interacts with HUVEC cells, although they remain unclear. Additionally, VM structures, an endothelium-independent pattern, increased in CEACAM6-overexpressing groups than in CEACAM6-silenced groups. Two methods were performed in this study to confirm this finding in GC. Both 3D-culture and PAS staining assays showed that CEACAM6 promoted VM formation in GC cells. The existence of VM structure is one phenotype of a malignant cancer. Furthermore, with the increasing tubule formation and VM structures in CEACAM6-overexpressing GC cells, nutrients were enough to be supplied for tumor growth. Consist with this inference, tumor volume and weight derived from CEACAM6-overexpressing groups were increased compared with the control groups *in vivo*. More interestingly, mosaic vessels were observed by mixing HUVEC and GC cells *in vitro*. This confirmed that nutrients and red blood cells could be transferred in VM vessels formed by GC cells as well as in tubules formed by HUVEC cells. These could strongly promote the progression of GC.

VM indicates a poor prognosis and defines highly invasive cancer phenotype. VEGFR-2 played a key role in VM formation in human glioblastomas [23]. In our study, we observed VEGF expression was increased in CEACAM6-overexpressing groups by nude mice tumor immunochemistry. To better understand a mechanism for how these processes could be regulated by CEACAM6, FAK a cytoplasmic tyrosine kinase, affecting cell survival, cell growth, angiogenesis, cellular invasion, and cellular migration [24–26], was detected in this study. Phosphorylation of FAK was increased in highly aggressive melanoma cancer cells than poorly aggressive cells, and increased FAK activity promoted VM and metastasis in melanoma [27,28]. FAK promoted tumor angiogenesis through the promotion of brain endothelial cell migration in malignant astrocytic tumors [25]. In accordance with these observations, we found that overexpressed CEACAM6 in GC cells and tissues increased phosphorylation of FAK and its downstream phosphorylation of paxillin. Additionally, the proliferation and migration of HUVEC cells were increased in CEACAM6-overexpressing groups and decreased in CEACAM6-silenced groups. Furthermore, the tyrosine 397, an autophosphorylation site of FAK, could be inhibited by Y15 inhibitor and is important in its downstream signaling functions.

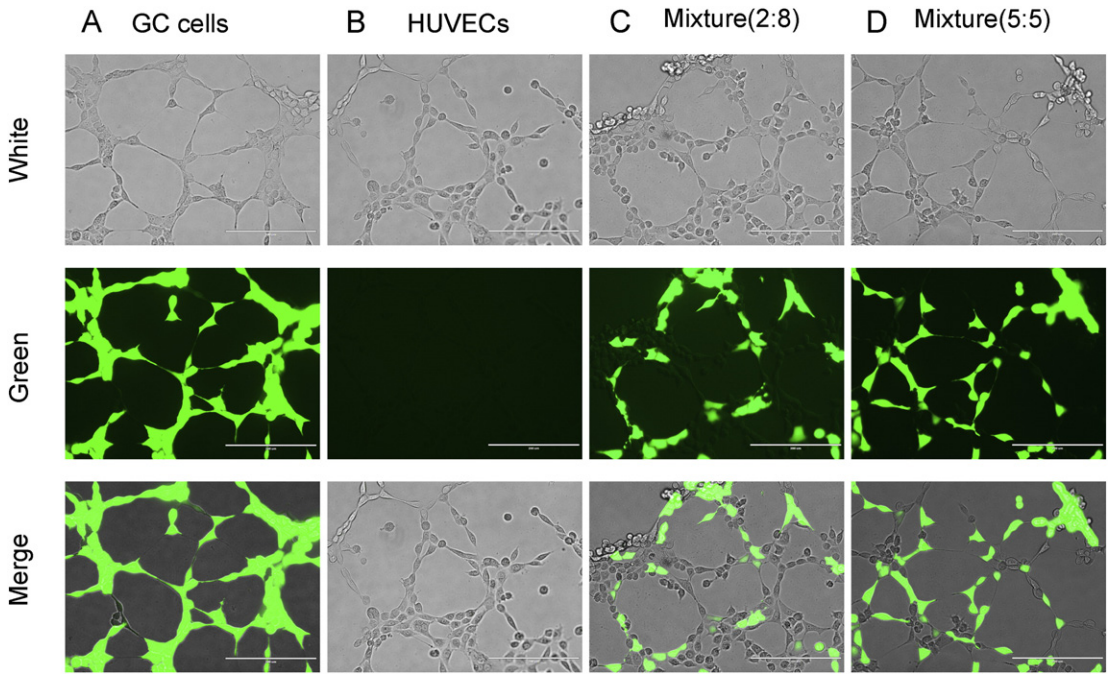

Fig. 5. GC cells and HUVECs form mosaic vessels on matrigel. (A) MKN-45-CEACAM6 GC cells with eGFP label formed VM on matrigel. (B) The tubules of HUVEC cells without eGFP label showed gray image under green light. (C) MKN-45-CEACAM6 GC cells mixed with HUVEC cells according to 2:8 (total cells 1×10^5) on matrigel. Mosaic vessels were observed under green light. (D) To clearly examine the mosaic vessels, MKN-45-CEACAM6 GC cells mixed with HUVEC cells according to 5:5 (total cells 1×10^5) on matrigel (400×). Green image: GC cells; gray image: HUVEC cells. Three independent experiments were performed.

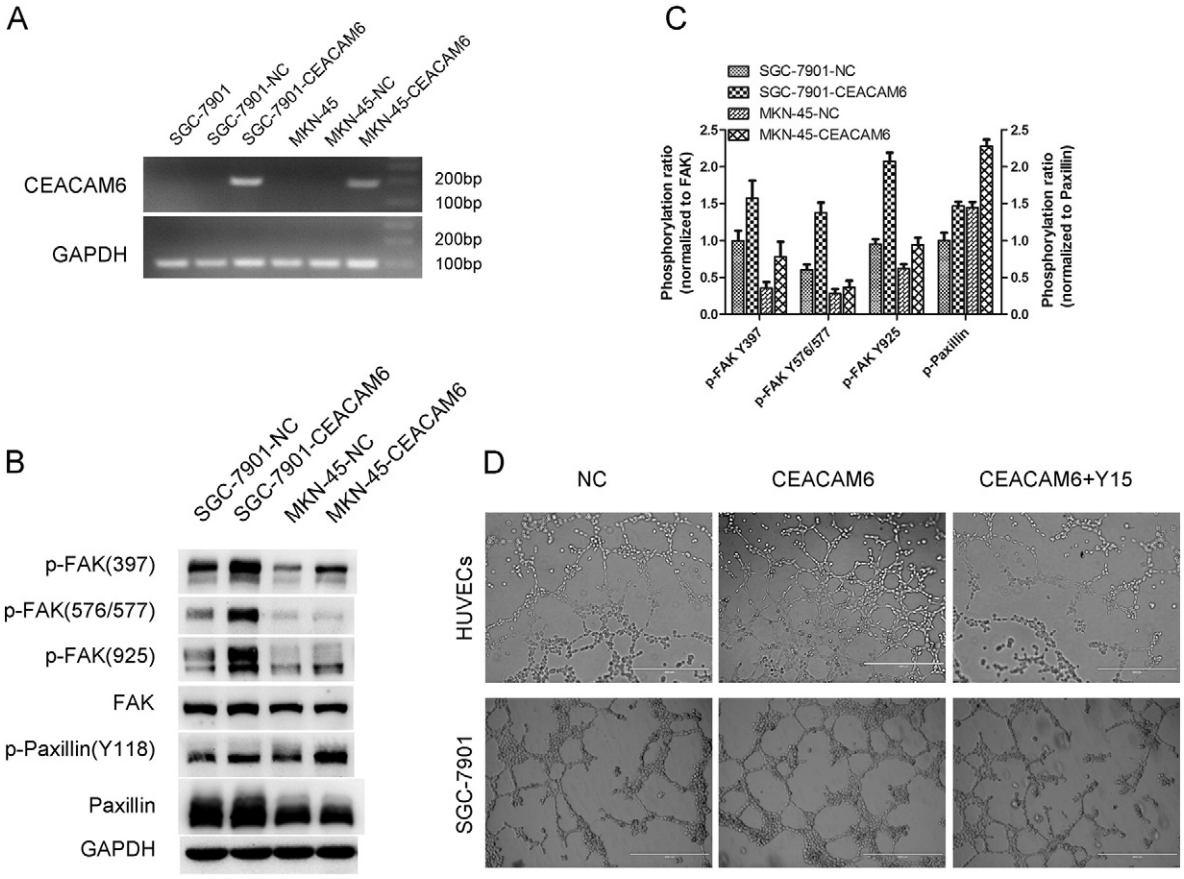

Fig. 6. CEACAM6 regulates FAK signaling in GC cells. (A) Overexpression of CEACAM6 in GC cells was confirmed at the RNA level by RT-PCR. (B) Phosphorylated FAK and paxillin were increased in CEACAM6-overexpressing groups compared with the control groups. (C) Histograms showed the phosphorylated FAK and paxillin ratio. Total FAK and paxillin as a loading control. (D) FAK inhibitor Y15 10 μM decreased tubule and VM formation (200×). Three independent experiments were performed.

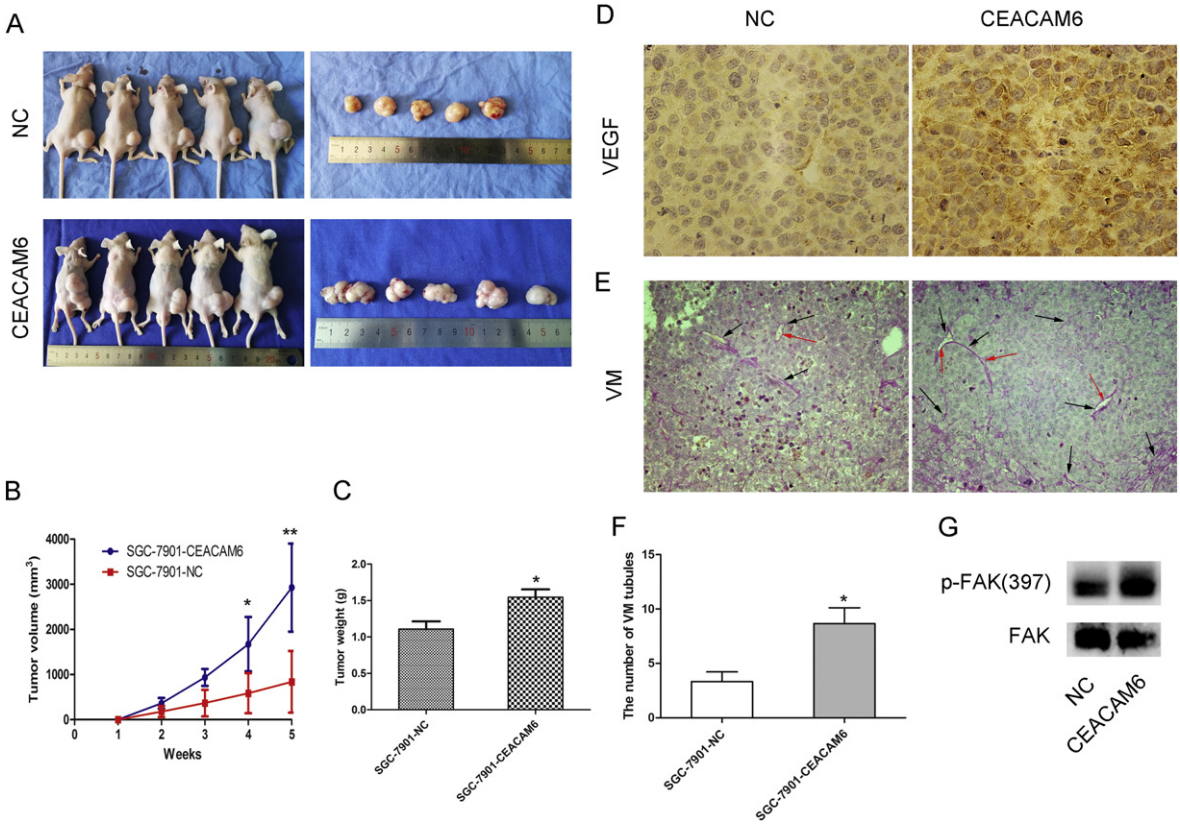

Fig. 7. Effects of CEACAM6 on tumor growth and VM *in vivo*. (A) Photographs of xenografts tumors of SGC-7901-NC and SGC-7901-CEACAM6. (B) Tumor volumes were measured every 7 days ($P < 0.01$). (C) Average weights of tumors in nude mice ($P < 0.05$). (D) Expression of VEGF in nude mice by immunohistochemistry (400×). (E) VM channels (black arrow) with or without red blood cells (red arrow) formed by GC cells were stained by CD34/PAS double staining (200×). (F) Histograms showed the numbers of VM tubules; $^{*}P < 0.05$. More VM structures were observed in SGC-7901-CEACAM6 groups than SGC-7901-NC groups. (G) Protein p-FAK (397) extracted from tumor of nude mice was increased in SCG-7901-CEACAM6 groups compared with the control groups.

Interestingly, we observed that Y15 decreased tubules and VM formation in CEACAM6-overexpressing GC cells. These findings suggested that CEACAM6 promoted GC angiogenesis and VM formation, at least in part, *via* FAK signaling. Moreover, we found that in our present study CEACAM6 induced epithelial–mesenchymal transition (EMT), which contributes to VM formation [29]. Collectively, CEACAM6 promoted angiogenesis and VM formation in gastric cancer *via* FAK signaling.

In conclusion, CEACAM6 promoted GC metastasis in our previous study [19] and stimulated tumor angiogenesis and VM formation in this study. These findings suggested that CEACAM6 may serve as a marker in GC defining cancer as a highly aggressive phenotype. As these effects of CEACAM6, we may identify a new avenue based on CEACAM6 for the GC therapy.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.02.005.

Conflicts of interest

The authors disclose no potential conflicts of interest.

Acknowledgments

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